Remarks

Support for the Amendments Status of the Claims

Claims 1-16, 93-101, 106-107, 112-133, 138-159, 162, 164-165, 167, 169, 171, 173-175, 177, 181-186, 188 and 193-197 are pending in the application, with claim 1 being the sole independent claim. Claims 12-16, 93-101, 106-107, 112-133, 138-140, 142-154, 162, 165, 167, 171, 175, 196-197 have been withdrawn from prosecution. Claims 17-92, 102-105, 108-111, 134-137, 160-161, 163, 166, 168, 170, 172, 176, 178-180, 187, 189-192, and 198 have been canceled without prejudice to or disclaimer of the subject matter therein. Applicants reserve the right to pursue any of the canceled subject matter in related applications. Claims 1, 164, 174, 182-186, 194 and 195 are sought to be amended. Support for the amendments to the claims can be found in the previous claims and in the application as filed. These changes are believed to introduce no new matter, and their entry has been respectfully requested.

Withdrawn Claims

Applicants respectfully thank the Examiner for confirming in the Office Action at page 3 that claims 173, 174, 177, 181-186, 188 and 192-195 have not been withdrawn from consideration.

Rejection Under 35 U.S.C. § 112, First Paragraph, Written Description

In the Office Action at pages 3-7, the Examiner has rejected claims 1-11, 141, 164, 169, 173, 174, 177, 181-186, 188, 193-195 under 35 U.S.C. § 112, first paragraph,

as allegedly failing to comply with the written description requirement. Applicants respectfully traverse this rejection.

In the Office Action at page 4, the Examiner asserts that the specification fails to provide adequate written description of 61 purified kinase and functional domains thereof from any organism such as mammals, bacteria, viruses.

In response to Applicants' arguments in the previous Reply to the Office Action filed April 20, 2009, the Examiner asserts that the numerous prior art documents cited by Applicants "teaches the numerous unforeseeable factors of a purified kinase positioned in an array… An applicant shows possession of the invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures and formulas to show that the invention is complete… Herein, kinase has been described only in words. The characterization of the different kinases from one organisms to another from the numerous kinases and numerous organisms has not been adequately described to distinguish one from the other." Office Action at pages 6-7.

Applicants respectfully disagree with these rejections.

The test for the written description requirement is whether one skilled in the art could reasonably conclude that the inventor had possession of the claimed invention in the specification as filed. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 U.S.P.Q.2d 1111, 1116 (Fed. Cir. 1991); M.P.E.P. § 2163.02. The Federal Circuit recently re-emphasized the well-settled principle of law that "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [they] invented what is claimed.'" *Union Oil Co. v. Atlantic Richfield Co.*,

208 F.3d 989, 54 U.S.P.Q.2d 1227 (Fed. Cir. 2000), hereinafter referred to as "*Unocal*." While the applicant must "blaze marks on trees," rather than "simply [provide] the public with a forest of trees," an Applicant is not required to explicitly describe each of the trees in the forest. *See Unocal*, 208 F.3d at 1000. The Court emphasized the importance of what the person of ordinary skill in the art would understand from reading the specification, rather than whether the specific embodiments had been explicitly described or exemplified. Indeed, as the court noted, "the issue is whether one of skill in the art could *derive* the claimed ranges from the patent's disclosure." *Unocal*, 208 F.3d at 1001 (emphasis added).

Applicants note that, as the Federal Circuit has held, the written description requirement must be viewed in light of the state of the art at the time of filing. *Capon v. Eshhar*, 418 F.3d 1349, 1357-1358 (Fed Cir. 2005) ("[t]he descriptive text needed to meet these [written description] requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence."). Furthermore, in *Capon*, the Federal Circuit stated that the Board's reliance on *Regents of the University of California v. Eli Lilly and Co.*, 119 F.3d 1559 (Fed. Cir. 1997), *Fiers v. Revel*, 984 F.2d 1164, 1169 (Fed. Cir. 1993), *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 927 F.2d 1200 (Fed. Cir. 1991) and *Enzo Biochem Inc., v. GenProbe, Inc.*, 296 F.3d 1316 (Fed. Cir. 2002), for the case at bar was incorrect and explained that "[n]one of the cases to which the Board attributes the requirement of total DNA re-analysis, *i.e., Regents v. Lilly*, *Fiers v. Revel*, *Amgen [v. Chugai]*, or *Enzo Biochem*, require a re-description of what was already known." *Id.* (emphasis added). Applicants submit, as noted herein and as argued previously, kinases and functional

kinase domains from yeast, mammals and *Drosophila* were a well characterized group of proteins that were generally known, understood to be well conserved in structure and function, easily identified, and readily prepared and assayed by those of ordinary skill in the art on the priority date of the present application. Thus, such proteins were well known to those of ordinary skill in the art, and hence, a re-description of such proteins is not required under *Capon*. *Id.*

Applicants respectfully submit, as previously noted, the level of skill and knowledge relating to protein kinases and their functional domains was very high on the priority date of the present application and a person of ordinary skill in the art would readily understand that indeed, Applicants were clearly in possession of a positionally addressable array comprising 61 purified active kinases or functional kinase domains thereof of a mammal, yeast or *Drosophila*. At page 26, lines 17-21 of the specification, it is stated that "[i]n a specific embodiment, one or more protein chips in the kit have, attached to the wells of the solid support, at least 50%, 75%, 90% or 95% of all expressed kinases... within the genome of an organism." The specification describes the use of positionally addressable arrays containing proteins and functional domains of the proteins from organisms including mammals, yeast and *Drosophila* (published [0058]); and provides a working example describing the production of a protein chip containing over 100 functional yeast kinases and yeast kinase domains (*See* Example I). In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the written description rejection under 35 U.S.C. § 112, first paragraph.

Rejection Under 35 U.S.C. § 112, First Paragraph, Enablement

In the Office Action at pages 7-13, the Examiner has rejected claims 1-11, 141, 164, 169, 173, 174, 177, 181-186, 188 and 193-195 under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. Applicants respectfully traverse this rejection.

The Examiner contends that while the present specification is enabling for Ser/Thr and Tyr kinases from yeast, it does not provide sufficient enablement of an array comprising 61 kinases and functional kinase domains of a mammal or *Drosophila*. The Examiner asserts that the presently claimed arrays encompass a broad genus of compositions because the present claims encompass any protein kinases from mammals, yeast and *Drosophila* and do not place any limitations on the kind, number and/or length of the kinases. Furthermore, the Examiner alleges that the specification does not provide any reasonable assurance that the 61 kinases found in yeast could be found in mammals or *Drosophila*, and that it is not apparent from the specification whether the same number of kinases or the kind of kinases or functional domain thereof can be found in any other organisms and made into an array. Finally, the Examiner asserts that in a highly unpredictable art, such as biotechnology, one cannot predict from a single species its correspondence or extrapolation to the genus. The Examiner therefore concludes that the presently claimed invention is not enabled. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As set forth in M.P.E.P. § 2164.01(a), there are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary

experimentation is "undue." These factors include the breadth of the claims; the nature of the invention; the state of the prior art; the level of one of ordinary skill; the level of predictability in the art; the amount of direction provided by the inventor; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *See In re Wands*, 858 F.2d 731, 737, (Fed. Cir. 1988).

As set forth in the Schweitzer Declaration at pages 3-4, section 8, and as previously discussed in the Snyder Deelaration and Replies to the Office Action filed December 21, 2007, and April 20, 2009, protein kinases and functional kinase domains used in the positionally addressable arrays that form the basis of the present claims were, at the time this application was filed, all well-known, and well-characterized. *See* Hunter and Plowman, "The protein kinases of budding yeast: six score and more," *TIBS 22*:18-22 (1997) at page 18, first column, first paragraph (cited in Applicants' 6th SIDS submitted on April 20, 2009). It was also well known at the time of filing of this application that kinases are highly conserved such that homologs exist between yeast and many other organisms. *See* Manning *et al.*, "The Protein Kinase Complement of the Human Genome," *Science 298*:1912-1934 (2002) at page 1913, first column, first paragraph (cited in Applicants' 6th SIDS submitted on April 20, 2009). Furthermore, the regulation of the different kinases and the phosphorylation motifs of substrates recognized by related kinases are often the same, indicating that they behave similarly biochemically. *See id.* Moreover, as the structure and function of kinases were known to be highly conserved, it was also known that human kinases can be substituted for yeast kinases, illustrating the highly conserved structure-function relationships known to

exist for kinases on the priority date of the application. *See* Lee and Nurse, "Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*," *Nature 327*: 31-35 (1987) (cited in Applicants' 6th SIDS submitted on April 20, 2009). Therefore, on the priority date of the present invention, the state of the art relating to protein kinases was extremely high and was such that a person of skill in the art, in the fields of for example, protein purification, proteomics and analysis, enlightened by the teaching of the specification would have appreciated that no more than routine experimentation would be required to make and use the claimed arrays containing purified active kinases or functional kinase domains from a mammal, yeast or *Drosophila*.

Applicants point out that it has already been acknowledged and accepted by Examiner Tran in the Office Action dated July 31, 2006, that "the large protein kinase superfamily are well characterized and known in the art such that the sequence of *any* kinases from *any* mammal, yeast and *Drosophila* can be determine[d] by bioinformatics tools and publicly available sequence information." Office Action at page 7, lines 4-7 (emphasis added). A person of ordinary skill in the art would readily recognize that any of the well known and characterized kinases, from any organism, could easily be utilized in the preparation of the presently claimed positionally addressable arrays. Therefore, on the priority date of the present invention, the "state of the art" in protein kinases was such that a person of ordinary skill in the art would have readily recognized from the present specification, and the knowledge available in the art, that kinases of yeast, mammals and *Drosophila* could routinely be utilized to practice the presently claimed invention.

Additionally, Applicants respectfully submit that methods useful for confirming kinase activity of the proteins on the claimed arrays are described in the specification and were otherwise well known as of the filing date of the present application (*see e.g.*, Example 1 of specification). *See also* Snyder Declaration at pages 3-4, section 7. Thus protein kinases, functional kinase domains and methods of assaying these proteins, were well-known in the art on the priority date of the present invention. The Examiner is reminded that, in order to enable a claimed invention, a specification need not teach, and preferably omits, information that is well-known to those of ordinary skill in the art. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986); *Lindemann Maschinenfabrik v. American Hoist and Derrick*, 730 F.2d 1452, 1463 (Fed. Cir. 1984); *In re Wands*, 8 USPQ2d 1400, 1402 (Fed. Cir. 1988). In addition, one of ordinary skill in the art is deemed to know not only what is considered well-known, but also where to search for any needed starting materials. *See In re Howarth*, 210 USPQ 689, 692, (CCPA 1981). Furthermore, "every aspect of a generic claim certainly need not have been carried out by an inventor, or exemplified in the specification," rather, what is needed is "reasonable detail" in order to enable members of the public to understand and carry out the invention. *Genentech, Inc. v. Novo Nordisk, A/S*, 103 F.3d 1361, 1366 (Fed. Cir. 1997).

As noted above, other factors to be considered when determining whether the claims are enabled by the specification are the amount of direction provided by the specification; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure. Applicants note that the present specification clearly provides numerous examples of methods for

preparing the presently claimed positionally addressable arrays utilizing yeast protein kinases, and as would be immediately apparent to a person of ordinary skill in the art, these methods are applicable and can if necessary, be routinely modified, for preparing the claimed arrays comprising mammalian or *Drosophila* protein kinases (*see e.g.*, specification at pages 27-38; *see also*, Snyder Declaration at pages 6-7, sections 11 and 12, *see also*, the Declaration of Barry Schweitzer at page 4, section 9 ("the Schweitzer Declaration") filed herewith.

In the Office Action at page 10, the Examiner contends that there is nothing in the Hanks disclosure to indicate that the hundreds of kinases disclosed therein are from mammals, *Drosophila*, or any other origin. *See Hanks, S.K. and Hunter, T., FASEB J.*, 9:576-596 (1995)(cited in Applicants' 6th SIDS submitted on April 20, 2009). The Examiner specifically contends that Hanks and the Snyder Declaration also fail to take into consideration the numerous factors of the claimed genus array besides the characterization of the kinases, such as how the kinases are purified and positioned on an array and how the kinases remain active. Applicants respectfully disagree for the reasons of record, and in addition to the disclosure provided in Hanks and the Snyder Declaration filed April 20, 2009, Applicants direct the Schweitzer Declaration, which describes the preparation of functional human protein kinase arrays using the teaching in the present specification. In addition, the functional human kinase domains used in the positionally addressable arrays prepared by Schweitzer that form the basis of the present claims were, on the priority date of the present application, well-known, well-characterized proteins with purified human kinases (*see* the Schweitzer Declaration, at page 3, section 8).

As discussed in detail in the Schweitzer Declaration at pages 4-7, sections 9-13, researchers enlightened by the information set forth in the specification, have used the homologies that were known to exist between human and yeast kinases, to informatically identify genes for human kinases and functional domains, clone these genes, express these genes in Sf9 insect cells, lyse the cells and purify the human kinases and functional domains. (*See also* Protein-Protein Interaction Profiling on Invitrogen ProtoArray™ High-Density Protein Microarrays, Application Note, Invitrogen page 2, column 2, paragraph 3 - 2, column 3, paragraph 1 (2005) (hereinafter "Protein-Protein Interaction Profiling," Exhibit B)). According to the Schweitzer Declaration, over 90% of protein kinases expressed and purified using the methods described in the specification were active as demonstrated by catalytic activity including autophosophorylation, wherein a protein kinase phosphorylates itself. *See id.* Finally, positionally addressable arrays as presently claimed have been manufactured with approximately 400 purified, active human kinases and functional kinase domains. *See* B. Schweitzer *et al.*, Development and Validation of Kinase Substrate Screening on Human ProtoArray High Density Protein Microarrays™, Invitrogen, Inc., page 2, column 1, paragraph 2 to page 3, column 1, paragraph 1 (2004) (hereinafter "Schweitzer") (Exhibit C). *See also* Access to the Human Proteome on a Microarray Scale, Invitrogen, Inc., Tables 1 & 2 (2007) (hereinafter "Access to Human Proteome") (Exhibit D). The activity of the arrayed kinases has been verified, including demonstrated catalytic activity by incubating the arrays with radioactive ATP and measuring autophosophorylation. *See* Schweitzer, page 2, column 2, paragraph 1 to page 3, column 1, paragraph 1. Thus, based on the high level of knowledge and skill in the art on the priority date, specifically, the ability to

identify and prepare purified functional protein kinases from yeast, mammals and *Drosophila*, the highly conserved nature of the proteins and the domains associated with kinase activity, and the detailed directions provided in the present specification, it would not have required undue experimentation for a person skilled in the art to prepare and use arrays comprising active kinases and functional kinase domains from a mammal, yeast or *Drosophila*.

The Examiner contends that, as the field of biotechnology is highly unpredictable, one cannot determine whether the generation of arrays comprising kinases of one organism (yeast) would be predictive of arrays comprising kinases of mammals or *Drosophila*.

Regarding these assertions, the Examiner is reminded that the present claims are directed to positionally addressable arrays, *not* proteins. The present specification provides detailed methods for attaching kinases or functional kinase domains to the surface of a solid support (*e.g.*, polydimethylsiloxane), for example, through the use of a 3-glycidooxypropyltrimethyoxysilane linker (GPTS). Applicants submit that the source or even identity of the kinase would not have any effect on the ability to attach the proteins to the surface of a solid support. The fact that a yeast kinase can be attached in this manner would clearly provide sufficient guidance to a person of ordinary skill in the art to utilize the same methods for attaching a kinase from a mammal or *Drosophila*, as discussed in the Snyder Declaration at pages 6-7, section 12.

As previously discussed in the Reply to the Office Action filed December 21, 2007, and April 20, 2009, Applicants agree with the Examiner that, prior to the disclosure of the present application, it was highly unpredictable to prepare positionally

addressable arrays comprising purified active kinases. It is only after the detailed disclosure of such methods in the *present specification* that such arrays could have been produced, as has been clearly demonstrated in the present specification. However, once these methods were provided, the ability to utilize these same methods to attach active kinases and functional kinase domains from organisms other than yeast was clearly not unpredictable. The Examiner is reminded that the test of enablement is not whether *any* experimentation is necessary, but whether, if experimentation is necessary, it is *undue. See* M.P.E.P. § 2164.01 (emphasis added). Applicants submit that at most, only routine experimentation would be required to prepare arrays of mammalian or *Drosophila* kinases or functional kinase domains. As noted above, kinases from yeast, mammals or *Drosophila* were easily identified and generated on the priority date of the present invention. Utilizing the detailed guidance provided in the present specification for attaching yeast kinases to a solid support, a person of ordinary skill in the art, with only routine experimentation, would have been able to prepare and use the claimed positionally addressable arrays comprising 61 purified active kinases or functional kinase domains from yeast, mammal or *Drosophila*.

The present application presents a situation very similar to in *In re Wands* where the specification was found enabling for the claimed antibodies because of the considerable direction and guidance in the specification, the high level of skill in the art, and the well-established methods needed to practice the invention. The present specification, like *In re Wands*, provides more than ample guidance to those of ordinary skill in the art for how to make and use the claimed arrays.

Accordingly, Applicants submit the present specification clearly enables the presently claimed invention. Reconsideration and withdrawal of this rejection are therefore respectfully requested.

Rejection Under 35 U.S.C. § 112, First Paragraph, Indefiniteness

In the Office Action at pages 12-13, the Examiner has rejected claims 1-11, 141, 164, 169, 173, 174, 177, 181-186, 188, and 193-195, under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Applicants respectfully traverse this rejection.

The Examiner has rejected claim 1 as allegedly being indefinite, stating that the phrase "functional kinase domain," is unclear. More particularly, the Examiner alleges "functional kinase domains" is vague and indefinite as to the kind, length or region the domain encompasses in a purified, active form to be a functional kinase.

Applicants respectfully disagree. Preliminarily, Applicants point out that as the M.P.E.P. 2173.05(b) instructs, the fact claim language may not be precise, does not automatically render the claim indefinite under 35 U.S.C. § 112, second paragraph. *Seattle Box Co., v. Industrial Crating & Packing, Inc.*, 731 F.2d 818, 221 USPQ 568 (Fed. Cir. 1984). Acceptability of the claim language depends on whether one of ordinary skill in the art would understand what is claimed, in light of the specification. Similarly, the Federal Circuit has held that claim dimensions need only be provided in terms that are "as accurate as the subject matter permits." *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 806 F.2d 1565, 1576 (Fed. Cir. 1986).

Applicants submit that the phrase "functional kinase domain" as used in the specification and claimed is not indefinite and that a person of ordinary skill in the art would understand that protein domains can rarely be delimited by absolute amino acid boundaries. In the present case, a person of ordinary skill in the art would understand that the claimed arrays comprise at least 61 active protein kinases and/or portions of protein kinase that retain kinase activity (*i.e.,* "functional kinase domains") with each protein kinase and/or functional kinase being at a different position on the array. Therefore, applicants submit that the phrase "functional kinase domains" is not indefinite.

With respect to the Examiner's rejection of claims 2-10, Applicants point out that the Examiner has provided no basis for rejecting these claims and Applicants respectfully request that this rejection be withdrawn.

The Examiner has rejected claim 11 as allegedly being indefinite, stating that the phrase "the solid support," lacks antecedent basis as claim 1 does not recite a solid support. Applicants respectfully disagree. Applicants respectfully point out that claim 1 is directed to "[a] positionally addressable array comprising a plurality of different substances on a *solid support*..." Thus, "the solid support" has antecedent basis in claim 1.

The Examiner has rejected claims 164, 169, 173, 175 and 177 as allegedly being indefinite, stating that the phrase "the organism" lacks antecedent basis. Claims 164, 169, 173, 175 and 177 have been amended to recite the term "mammal" in place of the term "organism." Thus, Applicants respectfully assert that the rejection is moot.

The Examiner has rejected claims 194 and 195 as allegedly being indefinite, stating that the phrases "the serine/threonine kinase family" and "the tyrosine family" lack antecedent basis. Applicants respectfully disagree. However, solely to advance prosecution, claims 194 and 195 have amended the claims to obviate this rejection.

The Examiner has rejected claim 174 as allegedly being indefinite, stating that claim 174 depends off of a cancelled claim. Applicants have amended claim 174 to depend from claim 164. Thus, Applicants respectfully assert that the rejection is moot.

The Examiner has rejected claims 181-186 and 193 as allegedly being indefinite, stating the term "organism" is broader than "mammals," "yeast" and "Drosophila" as recited in claim 1. Claims 181-186 and 193 have been amended to recite the term "mammal" in place of the term "organism." Examiner furthermore alleges that claim 1 recites 61 different kinases however, claims 182-186 recite 92, 110, 116, 119 and 122 purified active kinases, respectively, which the Examiner asserts is broader than the 61 purified kinases recited in claim 1. Applicants disagree with this claim construction, but nonetheless have amended claim 1 to recite the phrase "at least." Thus, Applicants respectfully assert that the rejection is moot.

In view of the foregoing remarks, Applicants respectfully submit that the rejections under 35 U.S.C. § 112, second paragraph, have been rendered moot or otherwise overcome. Reconsideration and withdrawal of this rejection are respectfully requested.

Rejection Under 35 U.S.C. § 102(a), or 35 U.S.C. § 103(a), Over Uetz

In the Office Action at pages 13-16, the Examiner has rejected claims 1-11, 141, 181-186, 188 and 193-195, as allegedly being anticipated by, or in the alternative, as allegedly being obvious in view of, Uetz *et al.*, *Nature 403*:623-631 (February 10, 2000) (hereinafter "Uetz"). Applicants respectfully traverse this rejection.

The Examiner contends that Uetz discloses a protein array comprising yeast genome encoded proteins, and that the proteins were expressed in 96-well plates. The Examiner asserts that the claimed kinases would have been inherent to the yeast array disclosed in Uetz, since yeast inherently contain kinase in their structure, or that they would have been obvious to determine given the identified genome of yeast. Applicants respectfully disagree that the claimed array is anticipated by Uetz.

Present claim 1 (and hence, claims 2-11, 141, 181-186, 188 and 193-195 that depend ultimately therefrom) recites a positionally addressable array comprising 61 *purified active* kinases or functional kinase domains thereof at a recited density. Applicants respectfully submit that Uetz does not disclose the preparation of an array comprising *purified active* kinases, and hence, cannot anticipate the presently claimed invention.

As discussed in Applicants' replies of December 21, 2007, and April 20, 2009, the Methods section of Uetz, at page 627, discloses that the arrays were prepared by transferring patches of transformed yeast cells into wells of a micro-array assay plate. Uetz does not disclose *any purification* of the yeast proteins prior to placement in the assay plate, just simply transfer of the transformed cells. Hence, Uetz does not disclose the use of *purified* kinases or functional kinase domains, as recited in claim 1. As set

forth in M.P.E.P. § 2131, "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of Cal.*, 814 F.2d 628, 631 (Fed. Cir. 1987). Thus, as Uetz does not disclose each and every element of present claim 1, it cannot and does not anticipate the presently claimed invention.

Applicants respectfully disagree with the Examiner's assertion that the Applicant's arguments relating to the composition of the array is not commensurate in scope with the claims, because the claims are drawn to an array and not to a method of making an array. Office Action, at page 15-16. Applicants agree that the presently claimed invention is indeed directed to an array (product) and point out that this product must comprise kinases (or kinase domains) that are *purified and active*. In other words, the recitations that the protein components of the array are in a *purified and active* state *characterize the components of the array*. Thus, as Uetz does not disclose arrays comprising *purified* kinases or functional kinase domains, clearly a required element of the claims, Uetz cannot and does not anticipate the presently claimed invention. The Examiner explicitly draws Applicants' attention to Uetz pg 623, col. 1, last paragraph – col. 2, first paragraph. Applicants respectfully assert that this disclosure provides further evidence that the arrays in Uetz did not consist of purified proteins having kinase activity.

With regard to the Examiner's assertion that Uetz renders obvious the presently claimed invention, Applicants note that, even assuming the arrays disclosed in Uetz comprise 61 kinases, there is no disclosure in Uetz sufficient to render obvious the

construction of an array of 61 kinases or functional kinase domains, in which the array comprises kinases that are *purified and active*, as recited in present claim 1.

The Examiner alleges on page 15 that Applicants' arguments as to the construction of the array are not commensurate in scope with the claims. The Examiner specifically reiterates that the claims are drawn to an array and not to a method of making the array. As above, Applicants agree with this point that the claims are drawn to an array and again respectfully assert that the limitation *purified and active* recited in the claims is not a process limitation, but rather a *characteristic of the components of the array*. Thus, the claims are directed to a "*positionally addressable array* comprising a plurality of different substances... wherein the plurality of different substances comprises at least 61 purified *active* kinases or *functional* kinase domains thereof..." (emphasis added)

As reaffirmed by the U.S. Supreme Court, courts are "to look at any secondary considerations that would prove instructive," when considering the obviousness of an invention. *KSR Int'l. Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1739 (April 30, 2007). For example, as set forth in M.P.E.P. § 2141(III), objective evidence or secondary considerations such as unexpected results and the skepticism of experts is relevant to the issue of obviousness and must be considered in every case in which they are present. Furthermore, as noted in M.P.E.P. § 2143.02, evidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness. *In re Rinehart*, 531 F.2d 1048 (CCPA 1976).

As noted in the December 21, 2007 reply, and the April 20, 2009 reply, at the time of filing of the present application, it was unexpected that kinases and their

functional kinase domains could be purified and placed on a solid support to form an array, and that these kinases and kinase domains *would retain their kinase activity*. As detailed in the Snyder Declaration and the Schweitzer Declaration, it is only after the guidance provided in the present specification that a person of ordinary skill in the art would consider it possible to generate the presently claimed arrays. As noted in the Snyder Declaration at pages 7-8, section 13, artisans in the field, before and even well after the time the present application was filed, thought that proteins in arrays, such as those used to prepare the arrays of the presently claimed invention, would denature and therefore be inactive. It was an unexpected and surprising result that the purified proteins on the arrays of the presently claimed invention retained their activity and could be utilized to determine meaningful biological interactions between the purified, active kinases and their targets (such as enzyme-enzyme or enzyme-substrate interactions). Applicants respectfully submit that, it is only after the guidance provided in the present specification that a person of ordinary skill in the art would consider it possible to generate the presently claimed arrays.

As discussed in the Snyder Declaration at pages 8-11, sections 14-19, the following exemplary references describe the skepticism from those in the field regarding the preparation of protein arrays both before and after the time of filing of the present application, as well as some of the problems regarding preparation of protein arrays comprising large numbers of purified active proteins that were overcome by the presently claimed invention.

For example, Anderson, K.S. and LaBaer, J., *Journal of Proteome Research* 4:1123-1133 (March 30, 2005) (copy provided as Exhibit A with Applicants' Reply to Office Action dated December 21, 2007; hereinafter "Anderson and LaBaer") state that:

> [t]heir theoretical advantages notwithstanding, protein microarrays have still not found widespread use, in part because producing them is challenging. Historically, it has required the high-throughput production and purification of protein, which then must be spotted on the arrays. Once printed, concerns remain about the shelf life of proteins on the arrays.

Anderson and LaBaer, page 1129. In addition, Shaw, G., *Drug Discovery and Development* (February 3, 2005) (copy provided as Exhibit B with Applicants' Reply to Office Action dated December 21, 2007; hereinafter "Shaw") states that:

> "[i]t was first thought that protein biochips would just be an extension of DNA microarrays, and that hasn't exactly panned out," says Bodovitz. That's because proteins have proven to be much trickier to work with in array format than their genomic counterparts. First of all, there are issues of stability. Membrane proteins, for example, make up the majority of potential drug targets, but they're particularly challenging to stabilize. Then there's the choice of immobilization technique, which determines how well the target protein presents itself to the capture agent, and the problem of nonspecific binding. And of course, proteins are inherently unstable outside their natural habitat of living cells, making them much more challenging than DNA to tag and manipulate.

Shaw at page 1.

In addition, as discussed in the Snyder Declaration and in the following references, it was well known in the art at the time of filing the present application that spotting proteins on solid surfaces often resulted in protein denaturation caused by uncontrolled adsorption, thereby inactivating the proteins. *See* Abstract of both Tleugabulva *et al.*, "Evidence for the denaturation of recombinant hepatitis B surface

antigen on aluminum hydroxide gel," *J. Chromatogr. B. Biomed. Sci.* 702:153-163 (1998) (cited in Applicants' 6th SIDS as NPL16); and Servagent-Noinville *et al.*, "Conformational Changes of Bovine Serum Albumin Induced by Absorption on Different Clay Surfaces: FTIR Analysis," *J. Colloid Interface Sci*, 221:273-283 (2000) (cited in Applicants' 6th SIDS as NPL15).

Thus, as noted in the references cited above, Applicants respectfully submit that prior to the filing of the present application, experts in the field were clearly skeptical of the ability to prepare protein arrays comprising purified active enzymes. In response to Applicants' arguments, the Examiner asserts that obviousness does not require absolute predictability, and asserts that as kinases were known at the time of filing, placing them on an array would have been obvious to one of ordinary skill in the art. Applicants note that the Examiner appears to be trying to have it both ways, now appearing to *agree* with Applicants that in fact, kinases of the various organisms such as mammals, *Drosophila* and yeast were well known at the time of filing the application. This is in stark contrast to the Examiner's contrary position noted above with regard to written description and enablement of the presently claimed invention.

While Applicants agree that kinases of *Drosophila*, yeast and mammals were well known in the art at the time of filing the present application, it would not have been obvious to place these kinases on a positionally addressable array so that they were not only *purified*, but also *active*. As discussed in the Snyder declaration at pages 9-13, sections 18-23, and in the references cited therein and below, prior to the presently claimed invention, only small numbers of proteins, and/or inactive proteins, at low densities, were able to be displayed on an array. It is only after the teachings of the

present application that large numbers of purified proteins were able to be placed on a solid support, in the recited density, such that they remained active.

For example, as discussed in Bussow *et al.*, "A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library," *Nucleic Acids Res. 26*: 5007-5008 (1998) (cited in Applicants' 6th SIDS as NPL3; hereinafter "Bussow1"), large scale protein arrays were produced, but only using denatured (and thus inactive) proteins. ("These protein filters were processed on pre-soaked blotting paper, i.e., denatured in 0.5 M NaOH, 1.5 M NaCl for 10 min, neutralized for 2x5 min in 1 M Tris-HCl, pH 7.5, 1.5M NaCl and incubated for 15 min in 2x SSC. Filters were air-dried an stored at room temperature." Bussow1 at page 507, second column, first paragraph.) Similarly, in Bussow *et al.*, "A human cDNA library for high-throughput protein expression screening," *Genomics 65*:1-8 (2000) (cited in Applicants' 4th SIDS as C9; hereinafter "Bussow2"), denaturing conditions were used to prepare the protein arrays. ("Twenty-five microliters of 50% Ni-NTA agarose was added to protein extracts obtained under denaturing conditions." Bussow2 at page 2, first column, third paragraph.) In addition, in the introduction of Bussow2, the authors discuss the difficulties in producing large amounts of purified proteins and maintaining their activity when placed on an array:

> the individuality of protein molecules demands highly customized procedures for their expression. Automation of these procedures requires systems that allow the efficient handling of large numbers of clones representing many different proteins. Bacterial systems are easy to manage but the expression of eukaryotic proteins can be problematic, due to aggregation, formation of insoluble inclusion bodies, and/or degradation of the expression product. Eukaryotic systems suffer from lower yields of heterologous protein (e.g. Saccharomyces cerevisiae), high demands on sterility (e.g

> mammalian systems) or time consuming cloning procedures (e.g. Baculovirus system).

Bussow2 at page 1, last paragraph bridging first and second columns (citations emitted, emphasis added). Thus, rather than attempt to overcome these various issues, the authors proceeded to produce denatured proteins in *E. coli* for use in their arrays. Similarly, Lueking *et al.*, "Protein microarrays for gene expression and antibody screening," *Anal. Biochem. 270*:103-111 (1999) (cited in Applicants' 6th SIDS as NPL11; hercinafter "Lueking"), also prepared protein arrays utilizing denaturing conditions (6M guanidinium-HCl, 0.1M NaH2PO4, 0.01 M Tris-HCl, pH 8.0, Lueking at page 104, second column, first paragraph).

In Ge, "UPA, a universal protein array system for quantitative detection of protein-protein, protein-DNA, protein-RNA and protein-ligand interactions," *Nucleic Acids Research 28*:e3(i-vii) (2000) cited in Applicants' 6th SIDS as NPL6; the author was only able to produce arrays comprising 48 proteins at a very low density, utilizing a traditional purification format. Extension of this disclosure to arrays comprising at least 100 different substances per cm^2 would have required extensive, undue experimentation beyond the scope of the disclosure provided in this reference.

Thus, as noted in the references cited above, Applicants respectfully submit that those of skill in the art were clearly skeptical of the ability to prepare protein arrays comprising large numbers of purified, active enzymes, at the densities recited in the presently claimed invention. Instead, denaturing conditions or traditional purification methods on small numbers of proteins were required.

Absent the teachings of the present application, there was no reasonable expectation that arrays comprising at least 100 different substances per cm^2, wherein the substances comprise 61 purified, active kinases on a solid support, could be successfully prepared, at the time of filing of the present application. It is only after the guidance of the present specification, specifically the methods described in the present application which allowed for large-scale purification and arraying of active kinases or functional kinase domains, that preparation of the presently claimed positionally addressable arrays was possible. The methods of the presently claimed invention, as discussed below and in the Snyder Declaration, are suitable for rapidly purifying large numbers of samples, such as the purification of active kinases isolated from mammalian, yeast, and *Drosophila*. As an example, kinases were purified by growing different strains of pep4 yeast cells, each containing a plasmid encoding a single GST-tagged kinase gene, in 96-well plates. Galactose was added to induce protein expression. The cultures of the same strain were combined, washed, resuspended and lysed. The GST fusion proteins were purified from these strains using glutathione beads and standard protocols in a 96-well format. This method allowed for the purification of a high number of yeast kinases in a relatively short amount of time. The buffers and methods used also ensured that the purified kinases or functional kinase domains retained their activity. *See* Example 1 of the present specification at page 26, line 25, through page 27, line 19.

Positionally addressable arrays were then prepared using the purified, active proteins purified as described above. Arrays were made from polydimethylsiloxane (PDMS) (Dow Chemical, USA), which was cast over microfabricated molds. Liquid PDMS was poured over the molds and, after curing flexible silicone elastomer array

sheets were then peeled from the reusable molds. Arrays were immersed in 3-glycidooxypropyltrimethoxysilane linker (GPTS) in order to facilitate adsorption of protein to the wells. To attach proteins to the chips, protein solutions were added to the wells and incubated on ice for 1 to 2 hours. After rinsing with cold HEPES buffer, the wells were blocked with BSA in PBS. *See* Example 1 of the present specification at page 27, line 21, through page 28, line 9.

As discussed above and throughout the Snyder Declaration, extending these methods to the preparation of arrays comprising purified active kinases from mammals and *Drosophila* was well within the level of the ordinarily skilled artisan and would not have required undue experimentation. Applicants respectfully submit that the methods set forth in the present application are sufficient to enable a person of ordinary skill in the art to make and use positionally addressable arrays comprising 61 purified, active kinases of yeast, mammals and *Drosophila*. However, such methods were only available as a result of the filing of the present application. It is only after the disclosure of the present methods that such arrays could be produced. The presently claimed invention is not simply a positionally addressable array comprising kinases, but rather, an array that comprises *purified active* kinases. There is no reasonable expectation of success that arrays of this type could have been produced, based on the disclosure of Uetz. Thus, Applicants respectfully submit that the presently claimed invention cannot be rendered obvious by the disclosure of Uetz.

In view of the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the rejections under 35 U.S.C. § 102(a) and 35 U.S.C. § 103(a). over Uetz.

Rejection Under 35 U.S.C. § 103(a) Over Shalon, In View of Felder or Lafferty

In the Office Action at pages 16-20, the Examiner has rejected claims 1-11, 141, 181-186, 188 and 193-195 under 35 U.S.C. § 103(a), as allegedly being upatentable over Shalon (WO 95/35505; hereinafter "Shalon") in view Felder *et al.* (U.S. Patent No. 6,458,533; hereinafter "Felder") or Lafferty (U.S. Patent No. 6,972,183; hereinafter "Lafferty"). Applicants respectfully traverse this rejection.

The Examiner contends that Shalon discloses a microarray having regions with a density of at least about $100/cm^2$, and that the arrays can comprise enzymes. The Examiner notes, however, that Shalon does not disclose arrays comprising kinases. The Examiner relies on the disclosures of Felder or Lafferty to cure this deficiency. Specifically, the Examiner contends that Felder discloses that kinases are enzymes, and Lafferty discloses an array containing substrate-enzymes, such as kinases. The Examiner therefore concludes that it would have been obvious to prepare the array disclosed in Shalon using the kinases disclosed in Felder and Lafferty, and hence, the presently claimed invention is rendered obvious. Applicants respectfully disagree with the Examiner's contentions and conclusions.

As stated in Applicants' replies of December 21, 2007, and April 20, 2009, and as discussed in the Snyder Declaration at pages 13-14, section 26, Shalon is primarily directed to arrays comprising polynucleotides (*see* Examples 1-3), and only mentions in passing that arrays comprising proteins and enzymes could be constructed. Furthermore, Felder discloses preparation of arrays comprising peptides that are *substrates* for kinases, not arrays comprising the kinases themselves: "[a] chimeric linker molecule is prepared in which a 25 base pair oligonucleotide complementary to one of the anchors is

crosslinked to a *peptide substrate of a tyrosine phosphokinase enzyme.*" Felder at column 44, lines 18-21 (emphasis added). Thus, Felder does not disclose the preparation of arrays comprising 61 purified active kinases or functional kinase domains thereof, as recited in present claim 1.

With regard to Lafferty, Applicants note that the arrays disclosed therein are limited to enzymes expressed in expression library cells, and that Lafferty does not disclose the purification of these enzymes prior to placement on a solid support, as recited in the presently claimed invention. As discussed in the Snyder Declaration, as set forth in Lafferty, at column 18, lines 1-14:

> The library comprises a plurality of recombinant clones, which comprise host cells transformed with constructs comprising expression vectors into which have been incorporated nucleic acid sequences derived from the DNA samples. One or more substrates and at least a subset of the clones is then introduced, either individually or together as a mixture, into capillaries (all or a portion thereof) in a capillary array. Interaction (including reaction) of the substrate and a clone expressing an enzyme having the desired enzyme activity produces an optically detectable signal, which can be spatially detected to identify one or more capillaries containing at least one signal-producing clone. The signal-producing clones can then be recovered from the identified capillaries.

Applicants respectfully submit, therefore, that Lafferty does not disclose arrays comprising 61 purified active kinases, as set forth in the presently claimed invention.

In view of the foregoing remarks, and those in the Snyder Declaration at pages 13-15, Applicants respectfully submit that Shalon, Felder and Lafferty, alone or in combination, do not disclose the presently claimed positionally addressable arrays, specifically, arrays comprising 61 purified active kinases or functional kinase domains thereof, as set forth in present claim 1. Specifically, the references cited by the Examiner

do not disclose arrays comprising purified active kinases, as recited in present claim 1. Thus, Applicants submit that the Examiner has not set forth a *prima facie* case of obviousness, as there are clearly differences between the cited references and the presently claimed invention that have not been addressed by the Examiner.

In response to Applicants' arguments, the Examiner asserts that Felder is employed for its disclosure of known kinases, that Shalon teaches the array of enzymes, to which the specific enzyme kinase would be prima facie obvious to position therein, and that Applicants have mischaracterized the use of Lafferty. The Examiner concludes that the combined teachings of the prior art would lead one having ordinary skill in the art to the claimed array of purified kinase. Applicants respectfully disagree with the Examiner.

The Examiner states that when considering obviousness of a combination of known elements, the operative question is thus "whether the improvement is more than the *predictable use* of prior art elements according to their established functions." *KSR Int'l. Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1739 (April 30, 2007). However, as previously stated by Applicants on page 28-31 of this reply, courts are "to look at any secondary considerations that would prove instructive," when considering the obviousness of an invention. *KSR Int'l. Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1739 (April 30, 2007). For example, as set forth in M.P.E.P. § 2141(III), objective evidence or secondary considerations such as *unexpected results* and the skepticism of experts is relevant to the issue of obviousness and must be considered in every case in which they are present. Furthermore, as noted in M.P.E.P. § 2143.02, evidence showing there was *no reasonable expectation of success* may support a conclusion of nonobviousness. *In re*

Rinehart, 531 F.2d 1048 (CCPA 1976). Such evidence goes directly against the argument that the improvement is a *predictable use* of prior art elements according to their established functions.

Applicants again assert that the references cited by the Examiner do not disclose arrays comprising purified active kinases, as recited in present claim 1. As noted on pages 28-31, in the December 21, 2007, reply and the April 20, 2009, reply, at the time of filing of the present application, it was unexpected that kinases and functional kinase domains of these kinases could be purified and placed on a solid support to form an array, and that these kinases and kinase domains *would retain their kinase activity*. As detailed in the Snyder Declaration and the Schweitzer Declaration, it is only after the guidance provided in the present specification that a person of ordinary skill in the art would consider it possible to generate the presently claimed arrays.

As discussed above and in the Schweitzer Declaration, Applicants respectfully submit that at the time of filing of the present application, it was unexpected that kinases and functional kinase domains of these kinases could be purified and placed on a solid support to form an array. It was also unexpected that the purified kinases and functional kinase domains of these kinases would retain their activity when placed onto the array. It is only after the guidance provided in the present specification that a person of ordinary skill in the art would consider it possible to generate the presently claimed arrays. Thus, prior to the filing of the present application, there was no reasonable expectation of success of preparing an array comprising 61 purified active kinases or functional kinase domains of these kinases. At the time of filing of the present application, experts in the field were skeptical, and it was unexpected, that purified

kinases and functional kinase domains of these kinases could be placed on a solid support to form an array, and that these proteins would retain their activity.

Therefore, in view of the foregoing remarks, and those in the Schwietzer Declaration, Applicants respectfully submit that the disclosures of Shalon, Felder and Lafferty, alone or in combination, cannot render obvious the presently claimed invention. Hence, reconsideration and withdrawal of the rejection under 35 U.S.C. § 103(a) are respectfully requested.

Double Patenting Rejection

Applicants thank the Examiner for withdrawal of the double patenting rejection.

Conclusion

All of the stated grounds of rejection have been properly traversed, rendered moot or otherwise overcome. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn.

Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Date: May 7, 2010

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EXHIBIT A

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PROFESSIONAL EXPERIENCE

INVITROGEN CORPORATION (Now LIFE TECHNOLOGIES), Carlsbad, CA 2004 - Present
Director, Integrated Technologies, Molecular Biology Systems Division – 2009 to present
Director, Protein Analysis R&D - 2008
Director, Protein Array R&D and Site Leader - 2006 – 2007
Director, Protein Array R&D and Operations - 2004 - 2006
Current responsibilities include the oversight of programs which span the traditional segments of the Molecular Biology Reagent Business, particularly programs that integrate instrumentation with consumables. Previous responsibilities included oversight of R&D and Services for Invitrogen's Protein Analysis product lines, including protein separation technologies, Western technologies, mass spectroscopy, and protein arrays. Additional responsibilities included site leadership of the Protein Array Center in Branford, CT, including R&D, Services, Manufacturing, Quality, and Facilities functions. Other responsibilities include budget preparation and implementation, intellectual property management, and oversight of academic, government, and industrial collaborations and contracts. Also participating in technology and intellectual property evaluations, business development, grant preparations, community relations, and presentations at national and international meetings. Reporting to the Vice President, R&D of the Molecular Biology Reagents Businces Unit.

Leadership accomplishments include:

- Led transfer of all operations from Branford, CT to Carlsbad on-time, under budget, and without loss of revenue
- Led global launch of several new multimillion dollar products
- Championed Lean Six Sigma Black Belt and Green Belt projects
- Led ISO 9001 Certification of Branford Site
- Led successful completion of multimillion dollar Biodefense projects in partnership with the United States Army Medical Research Institute for Infectious Diseases (USAMRIID)
- Authored or co-authored 11 publications, including paper in *Nature*
- Inventor or co-inventor on 10 new patent applications
- Presented at 14 international scientific conferences.

PROTOMETRIX, INC., Branford, CT 2002 - 2004
Senior Director, Technology - 2003-2004
Director of Technology – 2002 – 2003
Fifth person to join start-up biotechnology company. Director of a research and development operation providing high-throughput gene cloning, protein expression, protein purification, and protein microarray manufacturing for products, services, and discovery. Additional responsibilities included leading product development teams, leading technology and intellectual property diligence reviews, presenting to investors, coordinating industrial collaborations, and managing prosecution of company intellectual property. Reported to the Vice President, R&D.

Leadership accomplishments included:

- Led the Protometrix technical and IP diligence team during the acquisition of the company by Invitrogen Corp.
- Led the commercial launch of the world's first functional protein microarray product.
- Established the 1st manufacturing facility for the production of protein arrays.
- Built highly skilled team of scientists, engineers, and informatics specialists
- Led the design and buildout of 14,000 s.f. state-of-the-art laboratory and company headquarters.

MOLECULAR STAGING, INC., New Haven, CT 1998 - 2002
Director of Proteomics - 2001 – 2002
Section Head - 1998-2000
Second person to join start-up biotechnology company. Director of a research and service operation providing high-throughput protein expression profiling data using proprietary protein microarray technology to academic, government, and corporate clients. Responsibilities included management of research personnel, budget preparation and implementation, business development, oversight of academic collaborators, preparation of publications and patent applications, presentations for investors, corporate partners and at national meetings. Four direct and 23 indirect reports. Reporting to Chief Operating Officer.

Leadership accomplishments included:
- Successfully launched the world's first microarray-based protein expression profiling service.
- Developed the world's most advanced manufacturing facility for production of antibody microarrays.
- 8 publications, including publication in Nature Biotechnology of 1st application of antibody microarrays for protein expression profiling.
- 1 issued patent, and 4 patent applications.
- Led and coordinated the design and buildout of 46,000 s.f. of state-of-the-art proteomics laboratory.
- Successfully moved an academic technology into an industrial setting, increasing sensitivity, robustness, and utility.
- Led project resulting in $9 MM equity investment by Fortune 100 Company.
- Gave technical presentations resulting in $40 MM 2nd round financing.

WALT DISNEY MEMORIAL CANCER CENTER, Orlando, FL 1994 - 1998
Division Director. Laboratory director of multidisciplinary research program in the structural biology of nucleic acids, proteins, and drugs involved in cancer and related diseases. Responsibilities included carrying out experiments and data analysis, project development, management of 15-20 research, administrative, and volunteer personnel, budget preparation and implementation, grant writing, preparation of publications, public relations, and mentoring of graduate, undergraduate and high school students.
Scientific Director Molecular Diagnostics Clinical Laboratory. Responsibilities included business plan preparation and implementation, management of technical staff, technical consultant, clinical research director, and physician outreach.

Leadership accomplishments included:
- Established and directed a program utilizing multidimensional nuclear magnetic resonance (NMR) spectroscopy, and computational chemistry to determine high-resolution structures of proteins, nucleic acids, and drug complexes for the purpose of chemotherapeutic development.
- Established and directed a laboratory utilizing the most advanced molecular techniques to diagnose infectious diseases, cancer, and inherited diseases for patients of Florida Hospital (2nd largest number of admissions in U.S.).

UNIVERSITY OF CENTRAL FLORIDA, Orlando, FL 1994 - 1998
Assistant Professor
Responsibilities included: Research, Florida Hospital liaison, committee service, mentoring of graduate and undergraduate students, taught courses in Principals of Modern NMR Spectroscopy, Special Topics in Drug Development, Advanced Biochemistry Laboratory

EARLIER POSITIONS: **Associate Research Scientist** (1991-1993), Yale University School of Medicine, and **Research Associate** (1990-1991), Memorial Sloan-Kettering Cancer Center

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OTHER EXPERIENCE

GLYGENIX, INC., Cheshire, CT 2005 – 2007
Member, Board of Directors. Glygenix, Inc. was established to benefit children born with Glycogen Storage Disease, Type 1 (GSD1.) Its goal is to help find a cure for this disease by raising monies for GSD1-related research.

THE EPISCOPAL CHURCH AT YALE, New Haven, CT 2000 - 2003
Member, Board of Governors. The Episcopal Church at Yale (ECY) is a full time ministry of the Episcopal Church to students, staff and faculty at Yale.The ECY is governed by a Board of Governors of the Episcopal Church at Yale Corporation which is the legal entity of the Corporation in matters of contracts and other transactions with other institutions such as Yale University.

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EXHIBIT B

Protein-Protein Interaction Profiling on Invitrogen ProtoArray™ High-Density Protein Microarrays

A powerful means of determining the function of a protein is to map its interactions with other proteins. A variety of approaches are available to study protein-protein interactions, including mass spectroscopy, and yeast two-hybrid methods (1). Yet these technologies have several drawbacks: they are time-consuming, require expensive and specialized equipment as well as considerable expertise to run the equipment, and utilize large amounts of sample. Several large-scale efforts to map protein-protein interactions using mass spectroscopy or yeast two-hybrid have been performed recently (2, 3). Interestingly, a comparison of the results of these studies shows little overlap between the interactions observed in each, suggesting that the accuracy or the coverage of the methods may be lacking (4).

Protein microarrays have introduced a new approach to identify and characterize protein interactions, providing the ability to rapidly identify new interactions between thousands of proteins in a single experiment (5). Since the location and identity of each protein on the array is known, interaction maps can be developed rapidly from iterative probings of protein arrays. Because a protein microarray experiment is performed within a day, and interactions are assessed in the context of thousands of other proteins, interaction profiling on microarrays can greatly accelerate the rate at which novel protein interactions are discovered. Additionally, the *in vitro* nature of protein microarray experiments permits control over probing conditions that affect protein interactions such as protein concentration, post-translational modifications, and presence of cofactors, which may not be possible with other methods such as yeast two-hybrid screening.

MacBeath and Schreiber were among the first to demonstrate the potential of protein microarrays in protein-protein interaction, biochemical, and drug binding studies. In this study, pairs of proteins that were known to interact with each other—protein G and the immunoglobulin (IgG), p50 and IκKα, and the FKBP12 binding domain of FKBP with the human immunophilin FKBP12—were shown to interact on protein microarrays (6). Although this study represented a critical milestone in the development of functional protein arrays, only a few proteins were analyzed and novel activities were not identified. Since this report, a series of publications have demonstrated that proteins can retain their expected interactions while immobilized on microarray surfaces. Espejo *et al.* demonstrated that protein interaction domains, such as Src homology (SH2), 14.3.3, forkhead-associated (FHA), PDZ, pleckstrin homology (PH), and FF domains arrayed onto nitrocellulose-coated microarrays retain function and specificity, interacting with their corresponding ligands (7). Newman and Keating have used microarrays to characterize binary coiled-coil interactions from human basic-region leucine zipper transcription factors (8). More recently, Ramachandran *et al.* used protein microarrays to map pairwise interactions among several human DNA replication initiation proteins (9). Finally, in what may be the most striking example of the power of protein microarrays, Michael Snyder and colleagues at Yale University reported the fabrication of an array containing the majority of proteins from the yeast proteome and the use of this array to identify a new binding motif for calmodulin (10).

Invitrogen ProtoArray™ Products

Invitrogen has recently introduced the ProtoArray™ Microarray Technology for studying molecular interactions on protein arrays. The ProtoArray™ products include the ProtoArray™ Yeast Proteome Microarray nc v1.0, which contains 4088 open reading frames (ORFs) from *Saccharomyces cerevisiae*, and the ProtoArray™ Human Protein Microarray nc v1.0, which consists of nearly 1,900 human proteins. All proteins are expressed as N-terminal glutathione-S-transferase (GST) fusion proteins, purified, and spotted in duplicate on nitrocellulose-coated 1 inch x 3 inch glass slides. Using ProtoArray™ Microarrays allows screening of target proteins of interest for interaction with thousands of proteins in as little as four hours. Detection on the arrays is sensitive—as little as 1 pg protein on the array can be detected with submicrogram quantities of probe protein—and reproducible.

To detect protein-protein interactions on ProtoArray™ Microarrays, the protein probe must contain a label or tag to visualize the interaction of the probe with array proteins. The extremely high affinity of the biotin-streptavidin interaction makes biotin-protein conjugation a preferred method for protein labeling. Invitrogen offers the ProtoArray™ PPI Complete Kit for biotinylated proteins, which contains a module for efficiently biotinylating small amounts of a protein as well as qualified reagents for blocking, washing, and detecting biotinylated protein probes with streptavidin conjugated to a fluorescent dye, Alexa Fluor® 647.

Another preferred method of detecting protein interactions on ProtoArray™ Microarrays is to use protein probes with an epitope tag and a labeled antibody against the tag. An example of such a tag is the V5 epitope, a 14 amino acid (GKPIPNPLLGLDST) epitope derived from the P and V proteins of the paramyxovirus SV5. Invitrogen offers several Gateway® expression vectors that allow the fusion of the V5-tag to a protein of interest. The ProtoArray™ PPI Complete Kit for epitope-tagged proteins from Invitrogen provides reagents for blocking, washing, and detecting a V5-tagged protein using an Anti-V5-Alexa Fluor® 647 Antibody developed specifically for this application.

This Application Note demonstrates the utility of Yeast and Human ProtoArray™ Protein Microarrays for detecting protein-protein interactions using the biotinylated or epitope-tagged protein probes.

Materials and Methods

Yeast Proteome collection: The yeast proteome collection was derived from the yeast clone collection of yeast ORFs generated by the Snyder laboratory as described by Zhu *et al.* (10). Each *S. cerevisiae* open reading frame (ORF) was expressed as an N-terminal GST-6xHis fusion protein in a yeast expression vector. The identity of each clone was verified using 5'-end sequencing and the expression of GST-tagged fusion protein by each clone was confirmed with Western immunodetection using an anti-GST antibody. After verifying that each clone expresses a protein of the expected molecular weight, the proteins (from 4,088 clones) were expressed and purified using high-throughput procedures (10).

Human protein collection: The majority of the human protein collection is derived from the human Ultimate™ ORF Clone Collection available from Invitrogen (see http://orf.invitrogen.com for more information). The human proteins were expressed in the Bac-to-Bac® Baculovirus Expression System (Invitrogen Cat. no. 10359-016, for more information on the Bac-to-Bac® Baculovirus Expression System, visit www.invitrogen.com). Each Ultimate™ ORF Clone (entry clone) consists of a human ORF cloned into a Gateway® entry vector. Each entry clone was subjected to an LR reaction with the Gateway® destination vector, pDEST™20 to generate an expression clone. The LR reaction mix obtained after performing the LR reaction was transformed into competent DH10Bac™ *E. coli* to generate a recombinant bacmid. The high molecular weight recombinant bacmid DNA was isolated and transfected into Sf9 insect cells to generate a recombinant baculovirus that was used for preliminary expression experiments. After the baculoviral stock was amplified and titered, the high-titer stock was used to infect Sf9 insect cells for expression of the recombinant protein of interest in 96 deep-well plates. Following a 3-day growth, the insect cells were harvested for purification. All steps of the purification process including cell lysis, binding to affinity resins, washing, and elution, were carried out at 4°C. Insect cells are lysed under non-denaturing conditions and lysates were loaded directly into 96-well plates containing glutathione resin. After washing, purified proteins were eluted under conditions designed to obtain native proteins. After purification, samples of the purified proteins were run in SDS-PAGE gels and immunodetected by Western blot. The gel images were processed to generate a table of all the protein molecular weights detected for each sample.

ProtoArray™ manufacturing: The protein purification process described above produces thousands of purified proteins ready to be printed on arrays. A contact-type printer equipped with 48 matched quill-type pins is used to deposit each of these proteins along with a set of control elements in duplicate spots on 1" x 3" glass slides. The printing of these arrays is performed in a cold room under dust-free conditions to preserve the integrity of protein samples and printed microarrays. Before releasing the protein microarrays for use, each lot of arrays is subjected to a rigorous quality control procedures, including visual inspection of all the printed arrays to check for scratches, fibers, smearing, etc. To control for the quality of the printing process, several microarrays are probed with an anti-GST antibody. Since each protein contains a GST fusion tag, this procedure measures the variability in spot morphology, the number of missing spots, the presence of control spots, and the amount of protein deposited in each spot.

Cloning, Expression, and Purification of Proteins (6xHis-V5-BioEase™-EK-protein fusions): Ultimate™ ORF clones were obtained as entry clones and L x R cloned into pET105 for expression in *E. coli*. For each ORF, plasmid DNA was transformed into BL21 Star™ (DE3) *E. coli* cells, which were plated on LB/Amp and grown overnight at 37°C. Several colo-

nies from each of the 12 constructs were picked from LB/Amp plates and transferred into 50 ml of LB Amp. Cultures were grown from 5 to 7 hours at 37°C until an OD_{600} of 0.5 to 0.6 was reached. Next, 50 µl of 0.1 M IPTG was added to give a final concentration of 100 µM, and these cultures were incubated overnight at 20°C. Cell lysates were prepared using the protocol described in the ProBond™ Purification Resin manual. Pellets were resuspended with 8 ml Native Binding Buffer; 8 mg lysozyme was added and lysed for 30 minutes on ice. Cells were then sonicated on ice with six 10-second bursts and then centrifuged at 3,500 rpm for 20 minutes. Lysate (8 ml) was loaded onto a column with 2 ml washed ProBond™ resin and incubated for 1–2 hours at 4°C. The column was washed with Native Wash Buffer followed by an elution with 10 ml Elution Buffer. The pooled fractions were dialyzed twice against 2 L PBS. All samples were concentrated on Millipore spin membrane cartridges (10,000 MW cut-off) to a final volume of 250–350 µl, and were brought to 5% glycerol by the addition of an appropriate amount of 100% glycerol. Samples were then quick-frozen in liquid nitrogen and stored at –80°C.

In vitro biotinylation of proteins: Human calmodulin (Upstate) was biotinylated using the protocol outlined in the ProtoArray™ Mini-Biotinylation Kit (Invitrogen). Briefly, protein was biotinylated at room temperature for 1 hour and the sample was applied to a gel filtration column to remove unincorporated biotin. Protein concentration and the extent of labeling was also assessed.

Alexa Fluor® 647-streptavidin based detection: The protein-protein interaction assay was performed using the protocol outlined in the ProtoArray™ PPI Complete Kit for biotinylated proteins (Invitrogen). Arrays were blocked with 1% BSA/PBST at 4°C for 1 hour. Proteins were diluted in probe buffer (1X PBS, 5 mM $MgCl_2$, 0.5 mM DTT, 5% glycerol, 0.05% Triton X-100, 1% BSA) to 5 or 50 ng/µl and added to arrays under a cover slip, HybriSlip (included in the kit). Proteins were incubated at 4°C for 90 minutes in a 50 ml conical tube and then transferred to an incubation/hybridization chamber (included with the kit). Arrays were washed three times with probe buffer. Subsequently, a solution of Alexa Fluor® 647-streptavidin (Invitrogen, 0.75 µg/ml) in probe buffer was added and incubated at 4°C for 30 minutes. Arrays were washed three times and dried.

Anti-V5-Alexa Fluor® 647 based detection: The protein-protein interaction assay was performed using the protocol outlined in the ProtoArray™ PPI Complete Kit for epitope-tagged proteins (Invitrogen). Arrays were blocked with 1% BSA/PBST at 4°C for 1 hour. Proteins were diluted in probe buffer to 5 or 50 ng/µl and added to arrays under a HybriSlip cover slip. Proteins were incubated at 4°C for 90 minutes in a 50 ml conical tube and then transferred to an incubation/hybridization chamber (included in the kit). Arrays were washed three times with probe buffer. Subsequently, a solution of anti-V5-Alexa Fluor® 647 conjugated antibody (Invitrogen, 0.25 µg/ml) was added and incubated at 4°C for 30 minutes. Arrays were washed three times and dried.

Data acquisition/analysis: The microarray was scanned with a GenePix® 4000B Fluorescent Scanner (Molecular Devices). Data was acquired with GenePix® Pro software (Molecular Devices) and processed using ProtoArray™ Prospector (a software tool developed by Invitrogen that automatically performs data analysis, see www.invitrogen.com/protoarray for details) or Microsoft Excel and Microsoft Access. Statistically significant signals on each protein array were identified. The significant signals are greater than or equal to a value that is determined by calculating the median plus three standard deviations (using signal minus background values for all non-control proteins) for all non-control proteins on the array. Interactors were defined as proteins having positive significance calls not observed on the appropriate negative control.

Results

Probing ProtoArray™ Yeast Proteome Microarrays with biotinylated yeast proteins: Four yeast proteins were biotinylated *in vitro* using the Invitrogen ProtoArray™ Mini Biotinylation Kit. As shown in Figure 1, all four proteins showed expected interactions when used to probe the ProtoArray™ Yeast Proteome Microarray and detected with Alexa Fluor® 647-Streptavidin Conjugate. Each of the identified interactions is well annotated in the literature using a variety of different approaches (see http://www.yeastgenome.org for further details). Note that the interactions shown in Figure 1 are reciprocal. Biotinylated Ybr109C (calmodulin) interacts with Yfr014C (calmodulin kinase) on the array, and biotinylated Yfr014C interacts with Ybr109C on the array; the same relationship is observed with the GTP binding protein

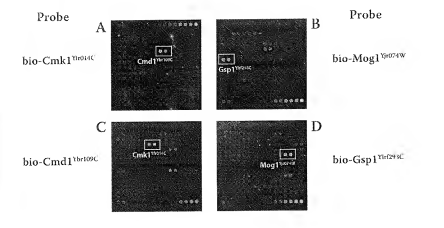


Figure 1—Probing the ProtoArray™ Yeast Proteome Microarray with *in vitro* biotinylated yeast proteins. Subarrays show expected interactions with biotinylated yeast proteins. Proteins were concentrated to 250 µg/ml and biotinylated using the ProtoArray™ Mini-Biotinylation Kit.

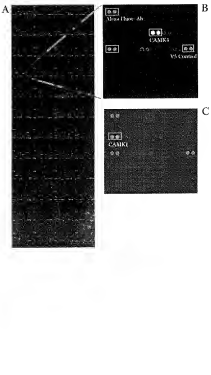
Gsp1 (Ylr293C) and the nuclear transport protein Mog1 (Yjr074W). The reciprocal interactions are important for demonstrating the validity of the observed interactions and the functionality of the proteins on the array.

Probing ProtoArray™ Human Protein Microarrays with Biotinylated and Epitope-tagged Human Proteins: To assess the utility of human protein arrays and protein-protein interaction detection technologies optimized at Invitrogen for demonstrating protein-protein interactions, proteins containing both a single biotin and a V5 tag were prepared (see Materials and Methods). Several N-terminal fusions of V5-BioEase™ human proteins were probed against human protein arrays (ProtoArray™ Human Protein Microarray nc v1.0) consisting of approximately 1,900 purified human proteins spotted in duplicate on a nitrocellulose-coated glass slide. After probing the array with calmodulin 2 (CALM2), we observed that CALM2 interacted with several proteins on the array. Most notable are the interactions with calcium/calmodulin-dependent protein kinase IV (CAMK4) and calcium/calmodulin-dependent protein kinase I (CAMK1) (Figure 2). These interactions were observed when streptavidin (data not shown) or anti-V5 based detection was used (Figure 2). We also used the ProtoArray™ Mini-Biotinylation Kit to *in vitro* biotinylate recombinant human calmodulin and used this protein to probe the ProtoArray™ Human Protein Microarray nc v1.0. As shown in Figure 3, similar protein interactions with CAMK1 and CAMK4 were observed for *in vitro* biotinylated calmodulin as with the BioEase™-tagged CALM2, demonstrating that valid protein-protein interaction data can be obtained by using proteins that are biotinylated using *in vitro* or *in vivo* methods.

To demonstrate the utility and ease of use of ProtoArray™ Technology for identifying novel protein-protein interactions, a V5-BioEase™ fusion to the protein cyclin-dependent kinase inhibitor 1B (CDK1NB, p27, Kip1) was used to probe a ProtoArray™ Human Protein Microarray. We identified a specific interaction with cyclin-dependent kinase 7 (Cdk7, MO15 homolog, *Xenopus laevis*, cdk-activating kinase) (Figure 4). The same interaction was also observed using streptavidin-based detection (data not shown). Although this interaction has not been reported previously in the literature, an interaction of CDK1NB with Cdk3 has been reported, and it has been proposed that retinoic acid induces cell cycle arrest in tumor cell lines by promoting formation of this complex (11). To validate the interaction, we performed the following reciprocal protein-protein interaction assay: CDK1NB was spotted on a nitrocellulose coated slide, then probed with GST-tagged Cdk7, and the Cdk7-CDK1NB complex was detected using an anti-GST antibody. Similar probings with 18 other GST-tagged proteins gave signals with the spotted CDK1NB that were on average approximately 10-fold lower than Cdk7 (Figure 5), indicating that the Cdk7-CDK1NB interaction is quite specific.

Summary

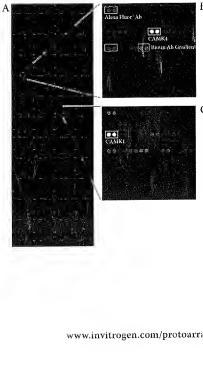
ProtoArray™ Protein Microarrays with Alexa Fluor® detection technologies are optimized to quickly identify novel protein-protein interactions. High-quality reagents, protocols and technical support are available. Consult the Invitrogen website for the latest information regarding protein microarrays for protein interaction profiling using ProtoArray™ Technology.



◄ **Figure 2—ProtoArray™ Human Protein Microarray nc v1.0 probed with human CALM2.** Interactions detected with anti-V5-Alexa Fluor® 647 Dye.

Panel A Whole slide image

Panel B Interaction of CALM2 with CAMK4. Signals from Alexa Fluor® Antibody and V5 control are shown. Alexa Fluor® labeled antibody is in every subarray and used as a reference marker for aligning the data acquisition grid. The V5 control is a V5 tagged protein printed on the slide. Signal with this protein indicates that assay detection is functioning properly.



► **Figure 3—ProtoArray™ Human Protein Microarray nc v1.0 probed with *in vitro* biotinylated human calmodulin.** Interactions detected with streptavidin Alexa Fluor® 647 Dye.

Panel A Whole slide image.

Interactions of human calmodulin with CAMK4 (*Panel B*) and CAMK1 (*Panel C*). Signals from Alexa Fluor® Antibody and biotinylated antibody gradient are shown. The biotinylated antibody gradient is used as assay detection control. Signal with this protein indicates that assay detection is functioning properly.

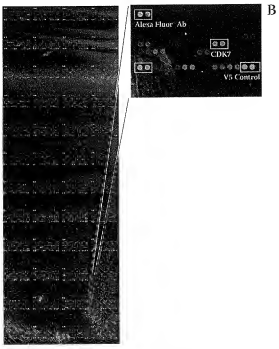


Figure 4—ProtoArray™ Human Protein Microarray 1c v1.0 probed with CDKN1B. Interactions detected with anti-V5-Alexa Fluor® 647 Dye. *Panel A* Whole slide image. *Panel B* Interaction of CDKN1B with CDK7. Signals from Alexa Fluor® Antibody and V5 control are shown.

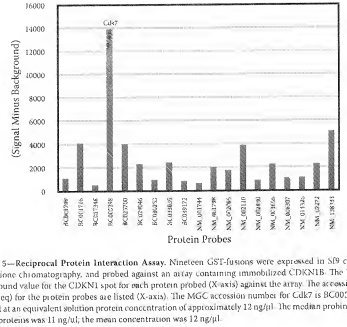


Figure 5—**Reciprocal Protein Interaction Assay.** Nineteen GST-fusions were expressed in Sf9 cells, purified using glutathione chromatography, and probed against an array containing immobilized CDKN1B. The Y-axis is the signal background value for the CDKN1 spot for each protein probed (X-axis) against the array. The accession numbers (MGC or RefSeq) for the protein probes are listed (X-axis). The MGC accession number for Cdk7 is BC005298. CDKN1B was spotted at an equivalent solution protein concentration of approximately 12 ng/µl. The median probing concentration for the 19 proteins was 11 ng/ul; the mean concentration was 12 ng/µl.

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EXHIBIT C

Development and Validation of Kinase Substrate Screening on ProtoArray™ High-Density Protein Microarrays

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Abstract

Identifying biologically relevant substrates for protein kinases is a critical step in understanding the function of these clinically important enzymes. Traditional approaches for kinase substrate identification are expensive, slow, and lack sensitivity. For this reason, many kinase activity assays employ generic substrates or peptides that decrease the reliability of these assays for drug development. We describe here the development and validation of a rapid and sensitive microarray-based kinase substrate identification technology, which enables parallel screening of kinases against thousands of potential native protein substrates. This paper describes the validation of this approach and use of the resulting data for pathway mapping.

Introduction

Protein kinases play a central role in the regulation of multiple cellular processes and in diseases; in fact, 244 kinases have been mapped to disease loci (1). It is not surprising, therefore, that a large number of biotech and pharmaceutical companies are seeking to discover and bring to the clinic compounds that demonstrate specific inhibition of kinases involved in disease. Some examples of kinase inhibitors already in clinic include Gleevec® (Novartis), an Abl and c-Kit kinase inhibitor that has been successful in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors, and Herceptin® (Genentech), an antibody that targets the HER2/neu (erbB2) protein for treatment of breast cancer. The family of human protein kinases consists of more than 500 members of which only a fraction have been characterized to date. Much is still not known about the biological function of many kinases, the protein substrates that are phosphorylated by these kinases, or the roles of these kinases and substrates in disease.

The importance of protein kinases in virtually all processes regulating cell transduction illustrates the potential for kinases and their cellular substrates as targets for therapeutics. Considerable efforts have been made to elucidate kinase biology by identifying the substrate specificity of kinases and using this information for the prediction of new substrates. Some of the approaches used to date include creation of a database from annotated phosphorylation sites, prediction of substrate sequence patterns from available structures of kinase/peptide substrate complexes, and screening of peptide libraries and peptide arrays (2,3). More recent efforts include attempts to map the phosphoproteome using mass spectroscopy-based techniques. While these studies have provided some information about kinase biology, they have been severely limited by their complexity, expense, lack of sensitivity, the use of non-structured peptides, and by poor representation of potential substrates in the screens.

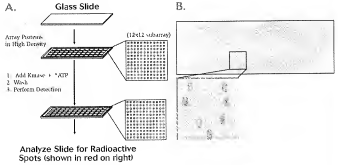
Invitrogen is pioneering the use of arrays of whole or partial proteomes to improve the success rates of drug discovery. This report describes how ProtoArray™ technology rapidly converts gene sequences into arrays of functional proteins that can be used to reveal new disease pathways and define the specificity and selectivity of potential drugs. In addition, this paper discusses how the ProtoArray™ high-density protein microarray technology is an ideal format for identifying biologically relevant substrates for protein kinases in a rapid, cost-effective, and comprehensive fashion.

Validation results

ProtoArray™ technology enables fast, simple, and comprehensive kinase substrate screening.

Each ProtoArray™ microarray contains thousands of *S. cerevisiae* or *H. sapiens* proteins spotted in high density on glass slides. These slides can be probed to identify protein interactions with DNA, proteins, lipids, sugars, small molecules, and enzymes. The first proof-of-principle experiment demonstrating that these arrays can be used to reveal substrates of proteins kinases was carried out on the Yeast ProtoArray™ microarray, which contains over 4000 unique yeast proteins spotted in duplicate. The experimental outline is simple (Figure 1A). A solution comprising a kinase and radioactive ATP was incubated on a Yeast ProtoArray™ microarray, and then the slide was washed and exposed to a phosphoimager (Figure 1B). The experiment identified 41 proteins specifically phosphorylated by the exogenous kinase.

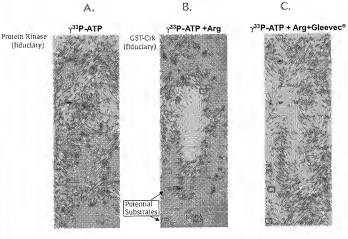
Figure 1. Kinase-substrate assay on the Yeast ProtoArray™ Microarray.



A) Experimental design of substrate screening assay. B) The Yeast ProtoArray™ microarray containing > 4000 different yeast proteins probed with a purified kinase. Inset: positives boxed in green (autophosphorylation) and red (substrates).

Initial work with Human ProtoArray™ microarrays demonstrates kinase substrate discovery value. To test our platform for identification of kinase substrates, we chose the human protein kinase Arg. This kinase, along with its closely related homolog Abl, is known to be involved in the etiology of chronic myeloid leukemia (CML) and is a target for the anti-cancer agent Gleevec®. Human ProtoArray™ microarrays were manufactured with 1500 different quality-controlled recombinant human proteins produced in Invitrogen's proprietary high-throughput insect cell expression and parallel purification systems. A known Abl/Arg substrate, Crk, was printed in regular intervals on the array as a positive control. The Human ProtoArray™ microarray in Figure 2A was incubated with radiolabeled ATP alone; proteins that show a signal on this array are kinases present on the array that autophosphorylate. The array in Figure 2B was incubated with Arg in the presence of radiolabeled ATP. This kinase phosphorylated the control substrate Crk in every subarray; in addition, nine other proteins, that did not give signal with ATP alone, were observed to be phosphorylated in the presence of Arg. We also looked at the effect of adding an Arg/Abl kinase-specific inhibitor and found that the inhibitor specifically decreased phosphorylation of Crk and the nine other microarray identified substrates (Figure 2C), confirming that these proteins were phosphorylated by Arg kinase.

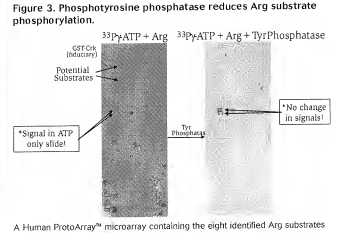
Figure 2. Identification of substrates for Arg kinase.



ProtoArray™ microarrays containing 1500 different human proteins were treated with ATP (A), ATP and Arg (B), or ATP, Arg, and Arg-specific inhibitor (C). Nine substrates were identified for Arg (boxed in red).

Verification of specific phosphorylation by a human kinase. Arg kinase is known to specifically phosphorylate tyrosine residues on certain proteins. To verify that Arg kinase maintains this specificity for tyrosine residues in array-based experiments, Human ProtoArray™ microarrays were treated sequentially with Arg kinase followed by a phosphotyrosine phosphatase. As shown in Figure 3, all proteins phosphorylated by Arg kinase on the array are dephosphorylated by the phosphotyrosine phosphatase, confirming that Arg kinase substrates on the array are appropriately phosphorylated on tyrosine residues. Signals from proteins that autophosphorylate (*i.e.*, that show signal in the absence of exogenous kinase) were not affected by phosphotyrosine phosphatase treatment, indicating that these were kinases that autophosphorylate serine/threonine residues.

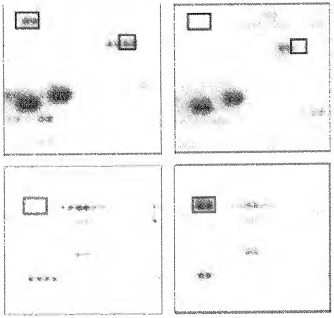
Figure 3. Phosphotyrosine phosphatase reduces Arg substrate phosphorylation.



A Human ProtoArray™ microarray containing the eight identified Arg substrates was probed with Arg kinase and then subsequently with a phosphotyrosine phosphatase.

Substrate phosphorylation is kinase-specific. The results with Arg kinase on Human ProtoArray™ microarrays clearly demonstrated that this kinase is highly selective in the protein substrates that it phosphorylates. In order for this application of the ProtoArray™ technology to be useful to a wide range of kinase biologists, the ability to distinguish phosphorylation patterns of different kinases must be established. Consequently, ProtoArray™ microarrays printed with 2500 different human proteins were incubated with ^{33}P-ATP and either Arg or PKC kinase (Figure 4) or with ^{33}P-ATP alone (not shown). As shown in Figure 4, phosphorylation signals specific to each kinase were clearly observed. The majority of signals present in both experiments were due to autophosphorylation by some of the ~400 kinases printed on the array. Analysis of the whole array revealed dozens of proteins that were specific to one of the kinases. We have now characterized the phosphorylation patterns of over a dozen different human kinases and have identified large numbers of unique substrates for each kinase.

Figure 4. Specificity of kinase phosphorylation on Human ProtoArray™ microarrays.

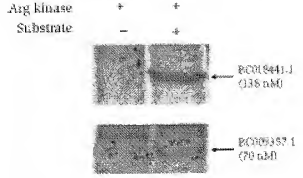


Two Human ProtoArray™ microarrays were incubated with ^{33}P-ATP and either Arg (left column) or PKC kinase (right column). Two representative subarrays are shown: In subarray 1 (top row), two proteins phosphorylated specifically by Arg kinase are boxed in blue; in subarray 2 (bottom row), a protein phosphorylated specifically by PKC is boxed in red.

Validation of substrate identification in an independent assay. Biochemical validation of the array-based substrate screening assay was initially carried out by determining whether proteins phosphorylated by Arg kinase on the array would also be phosphorylated in a different assay format. Figure 5 shows the results of assays in which two of the substrate proteins were incubated in solution with Arg kinase in the presence of radiolabeled ATP. Separation of the reaction mixtures on denaturing gels demonstrated that proteins at the expected molecular weight of the substrate proteins were indeed phosphorylated in solution. These results strongly suggest that these proteins maintain their native conformation on the array, allowing them to be phosphorylated by specific kinases.

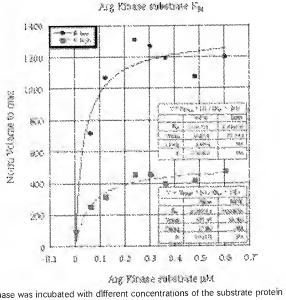
Detailed validation studies reveal the highest affinity substrate for a pharmacologically relevant kinase reported to date. Although phosphorylation of proteins by kinases in experiments, such as the one shown in Figure 5, is a prerequisite for identifying substrates for these enzymes, additional lines of evidence are needed to demonstrate physiological relevance. One such line of evidence is data showing that the substrate is phosphorylated at concentrations likely to occur in a cell. One of the eight proteins identified on the ProtoArray™ microarray as a substrate for Arg kinases was selected for more detailed K_M measurements based on the protein's known role in cell division. Analysis of the data from this experiment yields a K_M for the substrate of approximately 50 nM (Figure 6). Not only is this value well within a potential intracellular concentration for a protein, but it is also lower than any K_M value previously reported for Arg kinase.

Figure 5. Arg kinase phosphorylation of substrate in solution.



Arg kinase alone or mixed with substrate proteins was incubated at 30°C for 30 minutes and then run on an SDS-PAGE gel and phosphoimaged.

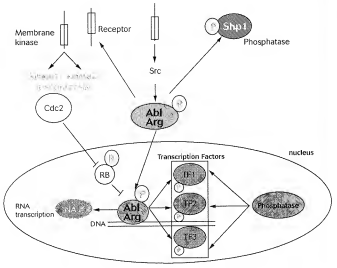
Figure 6. K_M determination for an Arg kinase substrate identified on the ProtoArray™ microarray.



Arg kinase was incubated with different concentrations of the substrate protein and the phosphorylation of the protein was measured in a gel-based assay similar to the one shown in Figure 4.

ProtoArray™ data is used to generate a new kinase pathway. In addition to biochemical validation, it is also desirable to see concordance of ProtoArray™ results with published data. In fact, a search of the literature and publicly available databases revealed that one of the proteins proven to be a substrate for Arg Kinase on a Human ProtoArray™ microarray, Shp1, had indeed been annotated as a substrate for this kinase. Using a protein-protein interaction assay on a Human ProtoArray™ microarray, we also demonstrated for the first time that Arg kinase forms a stable interaction with Shp1 (data not shown). Shp1 is a phosphotyrosine phosphatase localized at the plasma membrane; our data, as well as the published data, are therefore consistent with co-localization and co-regulation of Shp1 phosphatase and Arg kinase (Figure 7). Other published reports indicate that following activation by Src, Arg and Abl kinases translocate into the nucleus, although the functional consequences of this translocation have not been clarified. ProtoArray™ results, however, clearly showed that these kinases phosphorylated several transcription factors that may have roles in cell cycle function. An RNA polymerase was also phosphorylated, providing another line of evidence that these kinases regulate RNA transcription and gene expression. Equally intriguing is the finding that a membrane-associated receptor present on the array was phosphorylated by Arg kinase. Interaction of this receptor with a membrane-associated kinase has been shown by others to result in the activation of two kinases that have been implicated in oncogenesis. This finding represents a new and potentially therapeutically relevant link between the Arg/Abl kinases and cancer.

Figure 7. Pathway mapping with Arg kinase-substrate ProtoArray™ data.



Conclusion

We have combined unprecedented protein content with a simple-to-use microarray assay to generate new knowledge about protein kinases with unequalled efficiency. We have demonstrated specific phosphorylation of both known and novel substrates using Human and Yeast ProtoArray™ high-density protein microarrays and have validated these proteins as substrates using more standard assays. Combining this new type of information with Invitrogen's other capabilities for measuring phosphatase activity, protein-protein interactions, and drug inhibition on microarrays allows scientists to link kinases to intracellular signaling networks and generate new understandings about kinases and their substrates as drug targets with unmatched speed and efficiency.

Implications of ProtoArray™ technology

The discovery of new kinase substrates by Invitrogen and its collaborators using the ProtoArray™ technology platform demonstrates the enormous value of high-content protein arrays. This was clearly illustrated in experiments using Arg kinase: nine substrates were identified using an array printed with 1500 human proteins, but six more were found using a 2500 protein array. Extrapolating to an array containing a representative protein from the approximately 30,000 human genes (the UniProteome) suggests that over 150 substrates would be identified, thereby greatly increasing the informational value of the experiment.

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0-13121-r1 US 0704 xM

EXHIBIT D

Ordering information

Product	Quantity	Cat. no.
ProtoArray® Human Protein Microarray v4.0 *for protein-protein interactions (PPI)*	1 array	PAH0524101
ProLinker® Human Protein Microarray PPI Complete Kit *for biotinylated proteins*	1 kit	PAH0534011
ProtoArray® Human Protein Microarray PPI Complete Kit *for V5-tagged tagged proteins*	1 kit	PAH0534013
ProtoArray® Human Protein Microarray v4.0 *for molecular-response biomarker profiling (MRBP)*	1 array	PAH0524102
ProtoArray® Human Protein Microarray Discovery Study Bundle	20 arrays	PAH0534015
ProtoArray® Human Protein Microarray Discovery Study Bundle	40 arrays	PAH0534017
ProtoArray® Human Protein Microarray Discovery Study Bundle	80 arrays	PAH0534019
Immune Response Biomarker Profiling Application Kit	1 kit	PA0101
HuProt™ Control Protein Microarray v4.0 *for protein-protein interactions (PPI)*	1 array	PA0057
ProtoArray® Human Protein Microarray v4.0 *for kinase substrate identification (KSI)*	1 array	PAH0524006
ProtoArray® Human Protein Microarray KSI Complete Kit *for kinase substrate identification*	1 kit	PAH0534006
ProtoArray® Kinase Substrate Identification Application Kit	1 kit	PA0115
ProtoArray® Control Protein Microarray v4.0 *for known autoantigen identification (KAI)*	1 array	PA1002

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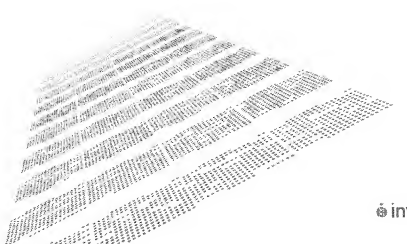
Access the human proteome on a microarray scale



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Access the human proteome on a microarray scale

ProtoArray® Human Protein Microarray v4.0

- Study >8,000 human proteins from multiple gene families
- Generate biologically relevant results by screening functional proteins
- Detect femtomole levels of protein from very small sample sizes
- Identify proteins immediately and access sequence-verified Gateway® clones for rapid follow-up validation

A high-quality human protein microarray with even higher content

The ProtoArray® Human Protein Microarray is an advanced, high-content, functional protein microarray (Figure 1) that enables you to scan thousands of proteins for biochemical interactions in as little as one day. Version 4.0 of the ProtoArray® Human Protein Microarray contains over 8,000 human proteins representing multiple gene families (Table 1), arrayed in duplicate on a 1 inch × 3 inch nitrocellulose-coated slide. The nitrocellulose array provides high protein stability and yields highly sensitive results for protein-protein interaction, kinase substrate identification, and serum profiling studies. In addition, each slide is labeled with a unique bar code for easy tracking.

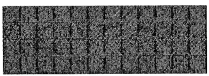

Figure 1—Anti-GST image of ... ProtoArray® Human Protein Microarray v4.0.

Maximum protein functionality

The ProtoArray® Human Protein Microarray is designed for maximum protein quality and functionality.[1] ProtoArray® microarray proteins are derived from sequence-validated open reading frames selected from Invitrogen's extensive Ultimate™ ORF Clone Collection.* These full-length proteins are expressed as N-terminal GST fusion proteins using a baculovirus-based expression system. All proteins are purified under nondenaturing conditions and printed at +4°C to preserve native structure and proper functionality.

Table 1—ProtoArray® content and protein distribution by class.

Class	No. on array[†]
Protein kinases	378
Transcription factors	188
Membrane proteins	1,085
Nuclear proteins	830
Signal transduction	710
Secreted proteins	100
Cell communication	863
Metabolism	2,166
Cell death	145
Protease/peptidase activity	138

† Numbers are based on Gene Ontology classification and are subject to change. For a complete list of array contents, visit [illegible]

* The Ultimate™ Human and Mouse ORF Clone Collection is the first and only clone resource to offer full-insert sequenced clones in a Gateway® vector. To learn more, visit [illegible]

Rapid and efficient data analysis

ProtoArray® Protein Microarrays can be easily read with most commercially available fluorescent microarray scanners. For a list of compatible scanners, please visit [illegible]. ProtoArray® Prospector v4.0 is a FREE data analysis software tool that evaluates results of control features present on the array and yields data obtained from experimental microarrays. You can download ProtoArray® Prospector v4.0 at [illegible]. Once you have identified your human proteins of interest, you can obtain the corresponding ready-to-use Ultimate™ ORF clones and get started on your downstream validation applications.

Broad range of discovery applications

The ProtoArray® Human Protein Microarray platform enables multiple discovery efforts (Table 2), including:

- Identification of novel protein biomarkers specific for infectious diseases, cancers, and autoimmune diseases[2]
- Mapping of protein-protein interactions important in biochemical pathways[3,4]
- Identification of kinase substrates for target discovery and validation[5]
- Antibody specificity profiling for research and therapeutic antibody development[6]

Table 2—The ProtoArray® Human Protein Microarray enables multiple ... applications ... with other approaches.

Application or attribute	Advantage	ProtoArray® Protein Microarray	Peptide arrays	Antibody arrays	Western blotting	Yeast two-hybrid	Mass spectrometry
Involves functional proteins	Biologically relevant results	Yes	No	Yes	No	Yes	Yes
Identified "hits" available as clones	Rapid follow-up validation	Yes, hits available as sequence-verified clones in Gateway® vectors	No	No	No	Yes	No
Low technical complexity	Easy to set up and integrate results	Yes	Yes	Yes	Yes	Yes	No
Protein-protein interaction discovery	Discover and validate drug target pathways	Yes, only 2 days	Limited	No	N/A	Yes, 6 weeks	Yes, 6 weeks
Antibody specificity profiling	Guide selection and development of research and therapeutic antibodies	Yes, detects cross-reactive species via linear and conformational epitopes	Fails to detect conformational epitopes, no specific identification of cross-reactive species	N/A	Fails to detect conformational epitopes, no specific identification of cross-reactive species	N/A	Possible
Biomarker discovery	Identify novel diagnostic markers for disease	Yes, autoimmune biomarkers only	Not demonstrated	Yes	N/A	N/A	Yes, coupled with labeling techniques
Profiling drug binding	Guide drug candidate selection and development	Yes	No	No	N/A	N/A	Yes